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PRINCIPAL INVESTIGATOR: Shaoyong Chen, Ph.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center Incorporated
Boston, MA 02215-5491

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Shaoyong Chen, Ph.D.

E-Mail: schen@bidmc.harvard.edu

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14. ABSTRACT:

This mid-term report is a summary of the work accomplished during the past research year. The majority of this work was included in the attached Manuscript. Additional data were also included. Together our data support a positive role of Pin1 in PCa progression. We demonstrated that Pin1 can enhance betacatenin nuclear localization, TCF/beta-catenin dependent promoter activity, and c-Myc and Cyclin D1 expression, while disrupt AR-mediated suppression of TCF/beta-catenin signaling. We also determined that Pin1 can reduce AR transcriptional activity and PSA expression, although the importance and the molecular basis for this pin1 action are still not clear.

15. SUBJECT TERMS
Prostate Cancer, Androgen Receptor, Beta-Catenin, Pin1, TCF

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Mid-term Report (PC040499, Postdoc Traineeship Award, Prostate Cancer Program 2004)
Title: Regulation of AR and b-catenin signaling by pin1 in prostate cancer
Shaoyong Chen, PhD; BIDMC, Harvard Medical School

INTRODUCTION:
The initiation of this project was emanated from our unexpected finding that the prolyl isomerase Pin1 could down-regulate beta-catenin's co-activation of AR, which is opposite to the stimulatory effects of Pin1 on TCF/beta-catenin signaling (1). Our interest was further inspired by the finding of a Texas group (2), which suggested Pin1 is overexpressed during PCa progression. In this mid-term report we briefly summarized of the work accomplished during the past research year. The majority of this work was included in the attached Manuscript. Additional data were also included in this report. Together our data support a positive role of Pin1 in PCa progression.

BODY: Our Task 1 (specific aim 1) is to test the hypothesis that Pin1 stimulates LNCaP cell growth by enhancing nuclear b-catenin accumulation and TCF signaling while repressing AR signaling. Our attack of this task is divided into 6 sub-projects from a) to f), as detailed below.

a), Generate LNCaP stable lines with increased Pin1 and TCF-4 expression.
We fully accomplished this part, by establishing stable LNCaP-Pin1 cell lines (see Manuscript); tetracycline inducible LNCaP-Pin1 cell lines (Figure 1), and tetracycline inducible LNCaP-TCF4 and dominant negative TCF4deltaN30 cell lines (Figure 2)

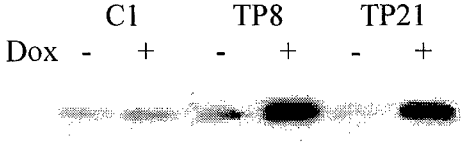

Figure 1. Tet-on-LNCaP-Pin1 stable lines have been generated. Tet-on LNCaP cells were transfected with either pTRE2-puro vector (*C1,* as control), or pTRE2-puro-Pin1 (*TP8* and *T21*).The cells are selected against puromycin. After treated with or without Doxycycline (Dox, 1 ug/ml), the cells were harvested in 1% SDS containing protease inhibitors. Protein was quantified with BCA assay and then equal amount of total protein was subjected to Western blotting with Pin1 (#07-091, Upstate).

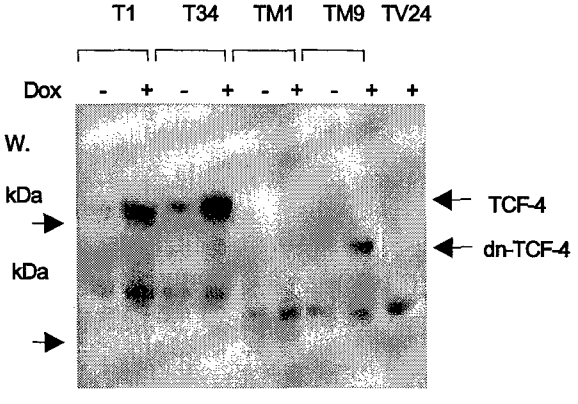

Figure 2. LNCaP stable lines were generated that express TCF-4 or dn-TCF4 in an inducible manner. Tet-on LNCaP cells were transfected with either pTRE2-puro vector (*TV24,* as control), pTRE2-puro-TCF-4 (*T1* and *T34*), or pTRE2-puro-dn-TCF-4 (TM1 or TM9). The cells are selected against puromycin. After treatment with or without Doxycycline (Dox, 1 ug/ml), the cells were harvested and equal amount of total protein was subjected to Western blotting with TCF-4 antibody (#6H5-3, Upstate).

b, Confirm that Pin1 increases nuclear b-catenin and selective coactivation of TCF in LNCaP cells by immunofluoresence and reporter assay.
Using con-focal immunofluoresence microscopy, we demonstrated that in Pin1-overexpressing LNCaP stable lines (see Manuscript), nuclear beta-catenin localization was dramatically increased in medium containing 10% of either charcoal-stripped serum (CSS) or normal fetal bovine serum (FBS) (Figure 3). We also used Luciferase reporter assay to confirm that pin1 increases TCF/beta-catenin dependent Top-flash reporter activity in CV-1, 293T, and LNCaP cells (see Manuscript).

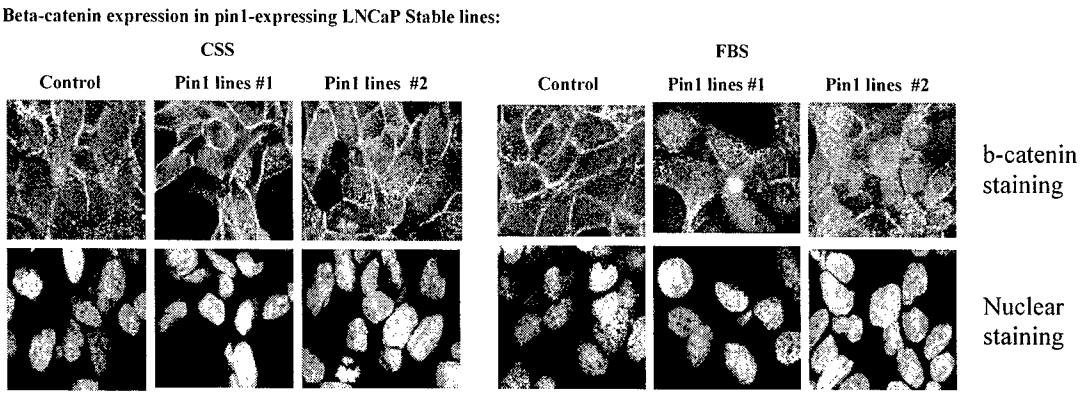

Figure 3. Overexpression of Pin1 in LNCaP led to enhanced nuclear beta-catenin. Pin1 stable LNCaP cells were grown in 24 well plate for 3 days, and then the medium was changed to medium containing CSS or FBS for 1 day incubation. Then cells were submitted for immunostaining and confocal microscopy.

c, Test whether Pin1 enhances the expression of endogenous genes regulated by TCF4 (c-Myc and cyclin D1), but not AR (PSA).
We demonstrated that increased expression of Pin1 in LNCaP cells led to enhanced c-Myc and cyclin D1 expression (see Manuscript). We also determined that transiently transfected Pin1 does not interfere with AR protein levels (see Manuscript), and increased Pin1 expression in LNCaP will not affect either total AR levels (Data not shown) or AR nuclear localization (Figure 4). However, induced Pin1 over-expression will down-regulate PSA expression (Figure 5A). This is consistent with pin1's inhibitory effects on AR transcriptional activity on both ARE4-Luc and PSA-Luc reporters (Figure 5B). The mechanisms of Pin1's negative effects on AR activity are under investigation.

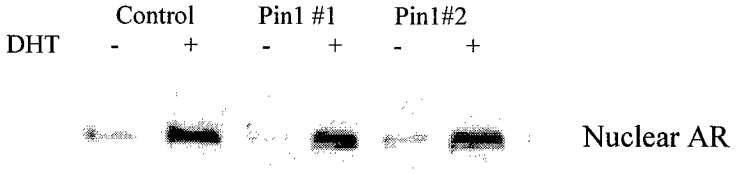

Figure 4. Pin1 Overexpression in LNCaP does not affect AR nuclear localization. LNCaP cells were grown in normal FBS medium for 2 days, and then the medium were changed to CSS medium and pulsed with 10 nM DHT. After 24 hr treatment, cells were fractionated with NE-PER kit (#78833, Pierce) and nuclear fraction is blotted with AR antibody (PG21, Upstate).

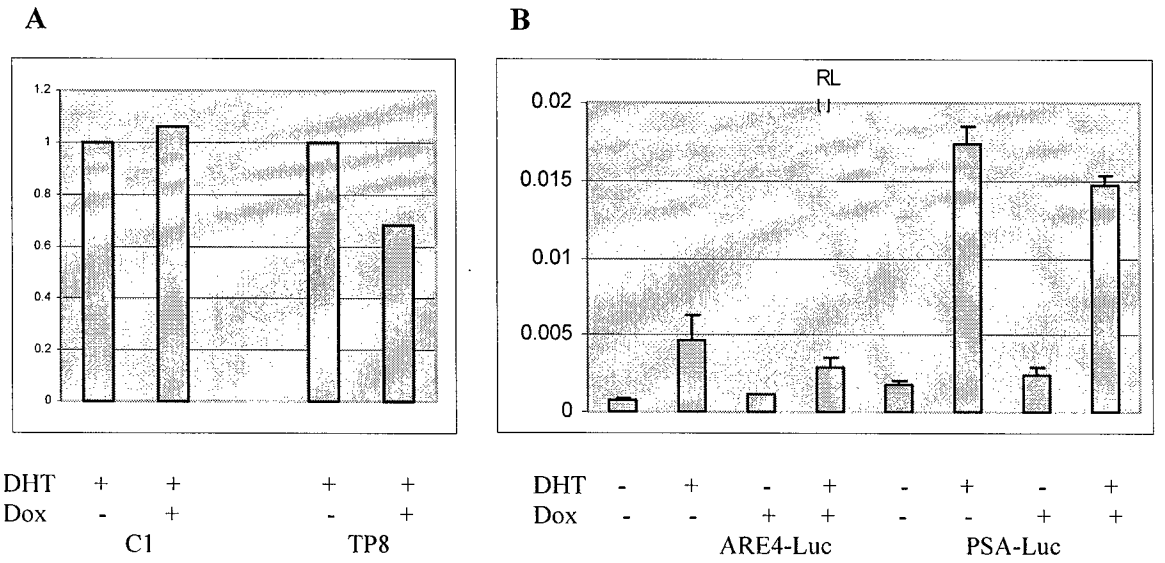

Figure 5. Induced Pin1 Overexpression in LNCaP leads to reduced PSA mRNA expression, which is consistent with the inhibitory effects on AR transcriptional activity. A) Tet-on stable LNCaP cells (C1 and TP8, see Figure 1) were grown in RPMI + 10% Tet-free FBS for 3 days. Then the medium were changed into RPMI + 5% CSS, with/without Dox (1ug/ml) for 24 hrs. DHT were added for 4 hrs and total RNA were isolated with TRIZOL reagent and submitted for real-time RT-PCR assay for PSA mRNA expression. B) TP8 cells were transfected with 50ng of ARE4-Luc or PSA-Luc. Then the medium were changed into RPMI + 5% CSS, with/without Dox (1ug/ml) and DHT (1 nM) for 24 hrs. Cells were harvested for Luciferase reporter Assay.

The sub-projects d), e), and f) are still under investigation.

Our Task 2 (specific aim 2) is to test the hypothesis that there is a distinct subset of prostate cancer expressing high levels of Pin1 and nuclear b-catenin. Our attack of this task is divided into 2 sub-projects a) and b). Part a) has been accomplished while part b) is still under investigation.

a, Examine pin1 and b-catenin expression patterns by immunochemistry on a series of primary and metastatic prostate cancer (paraffin sections and tissue arrays).

We determined by immunohistochemistry that the protein levels of pin1 are increased with the progression of prostate cancer (see Manuscript). However, we did not find a strong correction between total beta-catenin and pin1 levels, suggesting under PTEN-deficient circumstance like PCa, beta-catenin levels are regulated by additional mechanisms (see Manuscript).

b, Identify other b-catenin binding partners (like APC and E-cadherin) which may correlate with pin1 and b-catenin expression in prostate cancer.

This part is still under investigation.

KEY RESEARCH ACCOMPLISHMENTS:
1) A submitted Manuscript (see APPENDICES).

REPORTABLE OUTCOMES:
1) LNCaP cell lines stably express Pin1 (see Manuscript),
2) LNCaP cell lines stably express Pin1 in an inducible manner (see Figure 1),
3) LNCaP cell lines stably express TCF4 and dn-TCF4 in an inducible manner (see Figure 2),
4) SCID mice injected with LNCaP cell lines stably expressing Pin1 (see Manuscript).

CONCLUSIONS:
This mid-term report is a summary of the work accomplished during the past research year. As shown in the attached Manuscript, Pin1 promotes tumorigenesis in an animal model. We also showed by immunohistochemistry that pin1 expression is increased during PCa progression. As far as the data we have acquired, we contribute the positive role of Pin1 in PCa progression into the following mechanisms: First, as demonstrated in this report Pin1 can enhance beta-catenin nuclear localization; secondly pin1 stimulates TCF/beta-catenin dependent promoter activity and c-Myc and Cyclin D1 expression; thirdly, pin1 can disrupt AR-mediated suppression of TCF/beta-catenin signaling. However, we did not confirm the report (2) that pin1 can enhance total beta-catenin levels. An explanation for this discrepancy is that in PTEN deficient background, like in the case of most PCa, there is additional mechanisms involved in the regulation of beta-catenin stability.

In this report we also presented our preliminary data showing that Pin1 can reduce AR transcriptional activity and PSA expression. The exact molecular basis for this pin1 action is not clear, and we are at the beginning to address this issue. As shown above, we have determined AR expression and nuclear translocation is unchanged by overexpression of pin1. We also have preliminary data suggesting that pin1 may modulate AR N-to-C interaction. An additional mechanism may be AR phosphorylation. It is already known that AR phosphorylation is closely associated with AR transcriptional activity and PSA expression (3). The protein phosphatase 2A has been suggested to be involved in AR dephosphorylation (4) and Pin1 could facilitate the dephosphorylation activity of PP2A (5). Therefore a potential mechanism of Pin1 inhibitory effects on AR activity may be that pin1 can promote the AR dephosphorylation. Further study on AR phosphorylation/dephosphorylation will help answer this question. The significance of pin1's negative role on AR activity is not fully understood and also it is certainly important to know whether this pin1 action play a role in androgen-independence development.

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APPENDICES:
see attached submitted Manuscript.

Activation of β-catenin Signaling in Prostate Cancer by Peptidyl-Prolyl Isomerase Pin1 Mediated Abrogation of the Androgen Receptor-β-Catenin Interaction

Shao-Yong Chen,[1] Gerburg Wulf,[1] Xiao Zhen Zhou,[1] Mark A. Rubin,[2] Kun Ping Lu,[1] and Steven P. Balk[1*]

[1]Cancer Biology Program, Hematology-Oncology Division, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA

[2]Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA

[*]Corresponding author. Cancer Biology Program, Hematology-Oncology Division, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215. Phone:617-667-0878. Fax:617-667-5339. E-mail:sbalk@bidmc.harvard.edu.

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ABSTRACT

Androgen receptor (AR) interacts with β-catenin and can suppress its coactivation of T cell factor 4 (Tcf4) in prostate cancer (PCa) cells. Pin1 is a peptidyl-prolyl *cis/trans* isomerase that stabilizes β-catenin by inhibiting its binding to APC and subsequent GSK-3β dependent degradation. Higher Pin1 expression in primary PCa is correlated with disease recurrence, and this study found that Pin1 expression was markedly increased in metastatic PCa. Consistent with this result, increased expression of Pin1 in transfected LNCaP PCa cells strongly accelerated tumor growth *in vivo* in immunodeficient mice. Pin1 expression in LNCaP cells enhanced β-catenin/Tcf4 transcriptional activity, as assessed using Tcf4 regulated reporter genes, and increased expression of endogenous Tcf4 and c-myc. However, in contrast to results in cells with intact PTEN and active GSK-3β, Pin1 expression in LNCaP PCa cells, which are PTEN deficient, did not increase β-catenin. Instead, Pin1 expression markedly inhibited the β-catenin interaction with AR, and Pin1 abrogated the ability of AR to antagonize β-catenin/Tcf4 binding and transcriptional activity. These findings demonstrate that AR can suppress β-catenin signaling, that the AR-β-catenin interaction can be regulated by Pin1, and that abrogation of this interaction can enhance β-catenin/Tcf4 signaling and contribute to aggressive biological behavior in PCa.

INTRODUCTION

Prostate cancer (PCa) is the most common noncutaneous cancer in men in the United States and the second leading cause of cancer-related deaths in men in industrialized countries, but the molecular mechanisms involved in the development and progression of this disease are poorly understood. Nonetheless, many lines of evidence indicate that the androgen receptor (AR) functions as a positive regulator of cell proliferation in PCa, and androgen deprivation therapy is still the standard treatment for metastatic disease. AR is a member of the steroid hormone receptor subfamily of ligand regulated nuclear receptors, and its natural ligands are testosterone and 5α-dihydrotestosterone (DHT) (14). As with other steroid receptors, AR is a modular protein that contains an N-terminal transactivation domain, a conserved DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD). Ligand binding to the LBD induces conformational changes that generate binding sites for coactivator proteins, which stimulate transcription through chromatin remodeling and recruitment of the transcriptional machinery. One recently identified protein that can interact with and coactivate the AR is β-catenin, which binds to the DHT liganded AR LBD via a site that is distinct from the hydrophobic cleft that mediates binding of LXXLL motifs found in many other coactivator proteins (8,29,33,46,48,57).

β-catenin also functions in the nucleus as a transcriptional coactivator for the T-cell factor (Tcf) family of sequence specific transcription factors, and on the plasma membrane as a bridge molecule connecting E-cadherin to the cytoskeleton (17). Coactivator activity is determined by the level of free β-catenin, which is tightly regulated by a β-catenin degradation complex (18,21,36,41). This complex includes glycogen synthase kinase-3β (GSK-3β), the adenomatous polyposis coli gene product (APC), and Axin. APC binds to free β-catenin and recruits it to this

complex, where it is phosphorylated at N-terminal sites by GSK-3β and thereby targeted for ubiquitination and proteolysis. Wnt signaling stabilizes β-catenin by inhibiting GSK-3β activity, leading to increased cytoplasmic and nuclear β-catenin levels and activation of Tcf transcription factors. Tcf4 is the predominant Tcf in epithelia, and transcriptional targets of the β-catenin/Tcf4 complex include growth regulatory genes such as *c-myc* and *cyclin D1* (4,5,8,20,47,49,52).

The biological role of AR interactions with β-catenin has not been established, and may be complex given further direct interactions between AR and Tcf4, as well as between AR and amino terminal enhancer of split (AES, a Tcf corepressor and member of the Groucho/TLE family) (1,59). Although β-catenin can function as an AR coactivator and may selectively regulate a subset of AR responsive genes, another function for the AR-β-catenin interaction in normal prostate epithelium may be to sequester nuclear β-catenin and thereby suppress β-catenin/Tcf4 signaling, consistent with AR functioning in normal prostate epithelium to suppress growth and stimulate terminal differentiation (1,10,27,30,33,43,46). The vitamin D and retinoic acid receptors can similarly bind to β-catenin and interfere with Tcf4 coactivation by β-catenin (13,32,43).

The β-catenin/Tcf signaling pathway plays a critical role in normal development, stem cell renewal, and tumorigenesis. The importance of β-catenin/Tcf signaling in cancer has been most clearly demonstrated in hereditary colorectal cancer, where loss of APC leads to stabilization of β-catenin and increased expression of the β-catenin/Tcf4 target gene *c-myc* (20,28,49). Defects leading to β-catenin stabilization, including loss of APC or Axin function, or mutations in the N-terminus of β-catenin that prevent GSK-3β mediated phosphorylation, have been described in

sporadic colon cancer and in many other tumor types. β-catenin mutations have been identified in approximately 5% of prostate cancers, but a role for β-catenin in PCa development or progression has not been established (9,51). Nonetheless, immunohistochemical studies have shown increased cytoplasmic and nuclear β-catenin expression in 20-30% of PCa, with greater expression in more advanced tumors (8,11).

One mechanism for increased β-catenin expression in PCa may be PTEN loss, which is common in advanced PCa and results in activation of the PI3 kinase and downstream Akt signaling pathways (7,12,50). Akt can phosphorylate and inactivate GSK-3β, leading to stabilization and increased levels of β-catenin. Indeed, GSK-3β suppression and subsequent β-catenin stabilization have been demonstrated directly in the PTEN deficient LNCaP PCa cell line (34,44). However, LNCaP cells do not show substantial nuclear accumulation of β-catenin, and transfection studies with Tcf4 regulated reporter genes have shown minimal β-catenin/Tcf4 transcriptional activity, indicating that additional GSK-3β independent mechanisms may regulate β-catenin/Tcf4 activity in PCa (10,11).

An alternative mechanism for β-catenin stabilization is via Pin1 mediated proline isomerization, which can prevent β-catenin binding to APC (42). Pin1 is a peptidyl-prolyl *cis/trans* isomerase that targets phosphorylated Ser/Thr-Pro (pSer/Thr-Pro) peptide bonds, and has been found to regulate the activities of multiple proteins involved in cell cycle progression and other functions (23,24,37,56). The WW-domain of Pin1 appears to bind to pSer246-Pro247 in the third Armadillo repeat of β-catenin, with isomerization of this proline disrupting the interaction between β-catenin and APC (42). Overexpression of Pin1 has been implicated in cell

transformation and correlated with increased levels of β-catenin, cyclin D1, and c-myc in human breast cancer and other cancers (3,22,38,45,53-55). Significantly, Pin1 overexpression has also been observed in a subset of primary prostate cancers, and its expression correlates with increased risk of recurrence after radical prostatectomy (2). However, the functional effects of Pin1 overexpression on β-catenin nuclear signaling in PCa cells (and in particular in PTEN deficient cells), and how it contributes to more aggressive biological behavior have not been determined.

In this study we have assessed the role of Pin1 in regulating β-catenin activity in PCa. We found initially that Pin1 expression was markedly increased in metastatic versus primary PCa. Consistent with this result, increased expression of Pin1 in transfected LNCaP PCa cells strongly accelerated tumor growth *in vivo* in immunodeficient mice. The increased Pin1 expression in LNCaP cells enhanced β-catenin/Tcf4 transcriptional activity, as assessed using Tcf4 regulated reporter genes, and increased expression of endogenous Tcf4 and c-myc. However, in contrast to results in cells with intact PTEN and active GSK-3β, Pin1 expression in PTEN deficient LNCaP PCa cells did not increase the levels of total or free β-catenin. Significantly, while Pin1 expression in cells with intact PTEN could markedly enhance β-catenin coactivation of Tcf4, Pin1 expression markedly inhibited β-catenin coactivation of AR *in vivo* and AR binding *in vitro*. Moreover, Pin1 abrogated the ability of AR to antagonize β-catenin/Tcf4 binding and transcriptional activity. These findings demonstrate that Pin1 can regulate the AR-β-catenin interaction in prostate and contribute to aggressive biological behavior in PCa by abrogating this interaction and enhancing β-catenin/Tcf4 signaling.

MATERIALS AND METHODS

Plasmids and reagents. Expression vectors and reporter genes have been described previously (42). The AR LBD (amino acids 660–919) was cloned into the mammalian Gal4 DBD fusion vector pBIND (Promega), to give pBIND-AR-LBD. The AR DBD-LBD and AR N-DBD vectors were constructed in pcDNA3.1 (Invitrogen) and encode amino acids 501-919 and 1-500, respectively. GST-AR LBD encodes amino acids 676-919 of the AR LBD in the pGEX-2TK vector. Unconjugated anti-β-catenin was from BD Transduction Laboratories (San Jose, CA). Anti-AR (PG21), anti-Pin1 (07-091), and anti-Tcf4 (6H5-3) were from Upstate Biotechnology (Lake Placid, NY). FBS, charcoal-dextran stripped FBS (CDS-FBS), and tetracycline-free FBS were from Hyclone (Logan, UT).

Pin1 stable transfectants and xenografting. To generate Pin1 expressing LNCaP cell lines, pcDNA3.1 (control) or pcDNA-Pin1 plasmids were transfected into LNCaP cells and selected in medium containing 0.9 mg/ml G418. For *in vivo* growth, 2 million stable Pin1 transfected or control LNCaP cells were injected subcutaneously into the flanks of male ICR-*scid* mice (6-8 weeks, Taconic) in 50% Matrigel. Stable Pin1 and control clones derived from these lines were maintained in RPMI-1640 with 10% FBS and 0.3 mg/ml G418.

Immunostaining, immunoblotting, and real-time RT-PCR. Immunochemistry was done using tissue microarrays and primary antibodies at 1:20 for anti-β-catenin and 1:1000 for anti-Pin1. Free cytosolic and nuclear proteins were isolated with digitonin lysis buffer (1% digitonin, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$) containing protease inhibitors. Immunoblotting was carried out using the indicated primary antibodies, followed by HRP conjugates. Real-time RT-PCR was done with Taq-Man kits (PE Biosystem) and ABI Prism

7700 Sequence Detector (Perkin Elmer). The *c-myc* forward primer is: 5'-TGAGGAGACACCGCCCA-3'; reverse primer is: 5'-AACATCGATTTCTTCCTCA-3', and probe is: 5'-FAM-CACCAGCAGCGACTCTGA-3'. 18S rRNA was used as internal control.

GST pulldowns and coimmunoprecipitations. For GST pulldowns, 293T cells cultured in 10 cm dishes were transfected with LipofectAMINE 2000 (Invitrogen), as indicated. After overnight incubation, the cell culture medium was replaced with 10 ml DMEM containing 5% FBS. After another 24 hours, the cells were fractionated with the NE-PER kit containing protease and phosphatase inhibitors. The cytoplasmic protein fraction was precleared with glutathione-agarose beads (Amersham), and then equally divided and precipitated for 4 hours at 4°C with 20 µl of packed glutathione-agarose beads bound with GST or GST-AR-LBD fusion proteins (5 µg). For coimmunoprecipitations, 293T cells were transfected with 3 µg of each plasmid DNA as above. After overnight incubation, the cell culture medium was replaced with 10 ml DMEM containing 10% CDS-FBS and 10 nM DHT. After another 24 hours, the cells were lysed in binding buffer (PBS, 0.5% Triton X-100, 10% glycerol, and protease and phosphatase inhibitors). The cell lysates were pre-cleared for 20 min with 200 µg of nonimmune mouse serum absorbed on protein G-agarose beads. The supernatant was then split and immunoprecipitated for 1 hour with 1 µg of nonimmune mouse serum or anti-Tcf4 mouse monoclonal antibody absorbed onto protein G-agarose beads.

Transient transfections and reporter gene assays. CV1, 293T, or LNCaP cells cultured in 48-well plates were transfected using LipofectAMINE 2000, using the indicated amounts of each vector and with the addition of empty pcDNA3.1 vector to normalize for total DNA content (control experiments determined that inclusion of the empty vector did not affect specific or

control luciferase activities). Firefly and internal control *Renilla* luciferase activities were determined using a dual-luciferase reporter assay kit (Promega, Madison, WI), and *Renilla* activities were not consistently affected by any of the cotransfected vectors. The firefly luciferase was divided by the control *Renilla* luciferase and the results, given as relative luciferase units (RLU), reflect the mean and the standard deviation from triplicate samples.

RESULTS

Pin1 expression is increased in metastatic PCa. A previous study of Pin1 in PCa radical prostatectomy specimens found a correlation between higher levels of Pin1 expression and increased risk of PCa recurrence (2). Immunostaining on a series of normal prostate, primary and metastatic PCa samples showed that Pin1 expression was markedly increased in the metastatic tumors relative to the normal prostate (Fig. 1A-C and Table 1). Significantly, Pin1 expression in the metastatic PCa samples was also markedly increased relative to the primary PCa samples, with 24/29 metastatic tumors showing medium to strong Pin1 expression versus only 6/30 primary tumors showing medium staining (and none showing strong staining). Controls for immunostaining included anti-AR, which showed consistent nuclear expression in the epithelium (data not shown). This further increase of Pin1 in the metastatic versus the primary tumors was consistent with a role for Pin1 in metastatic behavior.

These metastatic tumors also showed increased cytoplasmic and nuclear β-catenin expression relative to the predominant plasma membrane expression in normal prostate epithelium (Fig. 1D). However, this increase was similarly observed in some primary PCa

samples with low or intermediate levels of Pin1, implicating additional factors (such as loss of E-cadherin and PTEN that occur frequently in PCa) in the altered β-catenin expression. Indeed, as Pin1 mediated proline isomerization stabilizes β-catenin by preventing its APC binding and subsequent GSK-3β mediated degradation, it was not clear to what extent increased Pin1 would enhance β-catenin activity or tumor growth in PCa cells with suppressed GSK-3β activity due to PTEN loss and Akt activation.

Pin1 expression enhances tumor growth and β-catenin/Tcf4 activity in PTEN deficient PCa cells. To determine whether increased Pin1 expression could enhance tumor growth and β-catenin activity in PTEN deficient PCa cells, we examined the PTEN deficient LNCaP PCa cell line stably transfected with a Pin1 or control expression vector. Immunodeficient male ICR-*scid* mice were implanted subcutaneously with Pin1 transfected (8 mice) or control transfected (4 mice) LNCaP cell lines in 50% Matrigel. As shown in figure 2A, growth of the Pin1 transfected cells was detected as early as 4 weeks after implantation (2 of 8 mice, 25%), with tumors in all 8 (100%) of the mice bearing Pin1 transfected cells by 6 weeks. In contrast, there was no detectable growth of the control transfected LNCaP cells at 6 weeks, with a small tumor detected in only 1 mouse (25%) at week seven. This difference was highly significant ($p<0.01$ by Fisher's exact test), indicating that Pin1 expression could enhance *in vivo* tumor growth.

A series of independent Pin1 or control LNCaP clones expressing varying levels of Pin1 were then generated and examined for β-catenin levels and for β-catenin coactivation of Tcf4 transcriptional activity. Significantly, there was no increase in the levels of total or free (digitonin soluble) β-catenin in a series of clones expressing varying levels of Pin1, including clones

expressing high levels of Pin1 relative to control LNCaP cells transfected with the vector alone (Fig. 2B). To assess β-catenin/Tcf4 transcriptional activity, clones were transfected with a Tcf regulated luciferase reporter plasmid (pTopflash). Consistent with previous reports, pTopflash specific activity was very low in control LNCaP cells (Fig. 2C). However, this activity was increased in Pin1 expressing clones. Similar results were obtained using a reporter gene derived from the *c-myc* promoter, which contains two previously characterized Tcf responsive elements (data not shown).

Expression of endogenous *c-myc* was next assessed to determine whether increased Pin1 levels enhanced expression of an endogenous β-catenin/Tcf4 target gene in PTEN deficient PCa cells. The expression of c-myc protein was increased in independent stable Pin1 transfectants compared to the vector alone LNCaP transfectant (control) (Fig. 2D). Real time RT-PCR confirmed that the increased c-myc protein reflected increased mRNA levels (Fig. 2E). Significantly, Tcf4 protein levels were also increased in the stable Pin1 transfectants. These findings indicated that Pin1 increased β-catenin coactivation of Tcf4, and suggested that enhanced β-catenin coactivation of Tcf4 resulted in selection for cells with increased Tcf4 levels and expression of β-catenin/Tcf4 regulated genes. Taken together, these studies showed that Pin1 could enhance β-catenin coactivation of Tcf4 in PCa cells by a mechanism that appeared to be distinct from its ability to increase β-catenin protein levels by suppressing β-catenin degradation.

Pin1 suppresses β-catenin coactivation of AR. Several groups have shown that β-catenin can also function as an AR coactivator, and that there may be cross-competition between AR and Tcf4 for limiting nuclear β-catenin (1,8,10,27,29,30,33,43,46,48,57). Therefore, a series of transfection studies were next carried out to determine how Pin1 mediated changes in β-catenin

affected Tcf4 versus AR activity. Consistent with previous data in Hela cells (42), Pin1 transfection enhanced the activity of the β-catenin/Tcf regulated pTopflash reporter gene in CV1 cells, and could further enhance the stimulation by cotransfected β-catenin (Fig. 3A). As shown in figure 3B, AR transcriptional activity could also be markedly enhanced by β-catenin transfection. However, in marked contrast to the Pin1 mediated enhancement of pTopflash activity, Pin1 suppressed AR activity on an ARE_4 reporter in the absence of transfected β-catenin, and completely abolished AR coactivation by transfected β-catenin (Fig. 3B).

To determine whether Pin1 inhibition of AR coactivation by β-catenin was dependent on a particular promoter context, we examined a luciferase reporter regulated by the androgen dependent promoter and enhancer from the PSA gene (PSA-Luc). As observed with the ARE_4 reporter, Pin1 suppressed AR activity and completely abrogated AR coactivation by transfected β-catenin (Fig. 3C). These opposite effects of Pin1 on AR and Tcf4 coactivation were not due to decreased AR protein expression and were similarly observed in transfected 293T, indicating that they were not cell type specific (data not shown). Finally, to assess whether Pin1 had a generalized inhibitory effect on steroid hormone receptors, we examined its effects on the estrogen receptor α (ERα). Consistent with previous data, β-catenin did not coactivate ERα activity on an ERE_2-Luc reporter gene .(Fig. 3D). Moreover, Pin1 transfection did not repress ERα transcriptional activity in the absence or presence of β-catenin. Taken together, these results indicated that the isomerization of β-catenin by Pin1 may prevent its interaction with AR.

Pin1 inhibits β-catenin interaction with the AR LBD. It was shown previously that β-catenin interacts with the AR LBD region, particularly helices 3, 5, 6, and 12 (46,57).

Therefore, to further test the hypothesis that Pin1 antagonizes the β-catenin-AR interaction, we examined the effect of Pin1 on β-catenin coactivation of the isolated AR LBD. The LBD was expressed as a fusion protein with the Gal4 DNA binding domain (pBIND-AR-LBD) and was tested using a Gal4 regulated luciferase reporter (pG5-Luciferase). The pBIND-AR-LBD protein had minimal transcriptional activity, consistent with previous data showing that this domain in the AR lacks a strong transactivation function (Fig. 4A). However, it could be strongly coactivated by transfection with β-catenin. As observed for the full length AR, Pin1 did not stimulate the LBD and completely antagonized the coactivation by transfected β-catenin.

Similar results were obtained when we examined the AR DBD-LBD, using an ARE_4-luciferase reporter. This construct was strongly coactivated by β-catenin, and Pin1 completely abrogated this activation (Fig. 4B). As a further control, we tested the effect of Pin1 on the transcriptional activity of AR N-terminus, which harbors a strong ligand-independent activation function (termed activation function-1, AF-1). As shown in figure 4C, β-catenin had no effect on AR AF-1 transactivation, confirming that β-catenin does not interact directly with the AR N-terminus. Importantly, AR N-terminus activity was not suppressed by Pin1, but was instead enhanced. This enhancement appears to be independent of β-catenin, and may reflect Pin1 effects on additional coactivators or corepressors.

Previous studies have shown that cyproterone acetate (CPA) functions as an AR partial agonist, and that the CPA liganded AR is not coactivated by β-catenin (1,27). Therefore, if the inhibitory effect of Pin1 on the DHT liganded AR is due to blocking β-catenin-AR interaction, then Pin1 should not antagonize the CPA liganded AR. To test this hypothesis, CV1 cells were transfected with AR, β-catenin and Pin1, and were then treated with DHT or CPA. Consistent

with previous results, β-catenin stimulated AR activity in the presence of DHT, but not CPA (Fig. 4D and E). Indeed, β-catenin had a modest inhibitory effect on the CPA liganded AR, which likely reflected sequestration of other coactivators. Importantly, the CPA-liganded AR was not inhibited by Pin1 in the absence or presence of exogenous β-catenin (Fig. 4E).

Taken together, these data indicated that Pin1 was inhibiting the interaction between the AR LBD and β-catenin. To determine whether this inhibition was dependent on the peptidyl-prolyl isomerase activity of Pin1, we examined a previously described catalytically inactive Pin1 mutant, K63A (42). CV1 cells were transfected with the AR LBD expressed as a fusion protein with the Gal4 DNA binding domain (pBIND-AR-LBD), β-catenin, and varying amounts of wild-type (WT) or K63A (KA) mutant Pin1. As shown in figure 4F, the wild-type Pin1 was more active than the K63A mutant at inhibiting β-catenin coactivation of the AR LBD, although the mutant also had inhibitory activity. Immunoblotting confirmed that the proteins were expressed at comparable levels. This result supports a role for the isomerase activity, while it is not yet clear whether the inhibitory activity of the K63A mutant reflects residual enzymatic activity or β-catenin blockade by binding to the Pin1 WW-domain.

AR LBD is not a direct target of Pin1. Although β-catenin has been shown to be a direct Pin1 target, it was possible that Pin1 abrogation of the AR-β-catenin interaction was due to a direct effect of Pin1 on the AR LBD. The AR LBD contains a single potential Pin1 target site, Thr799-Pro800, which lies in the kink between helices 7 and 8. The proposed Pin1 target site on β-catenin is similarly located in a kink between two helices in armadillo repeat 3 (42). Therefore, as AR is an extensively phosphorylated protein, phosphoThr799-Pro800 may serve as a Pin1 substrate. To test this hypothesis, we generated a Thr799Ala mutant AR and assessed the effects

of Pin1 on this mutant versus the wild-type AR. As shown in figure 5A and B, the wild-type and Thr799Ala mutant AR were similarly stimulated by DHT and coactivated by β-catenin. Significantly, Pin1 suppressed the activity of the T799A mutant and abrogated its coactivation by β-catenin (Fig. 5B). These data indicate that Pin1 inhibition of the AR-β-catenin interaction is mediated through β-catenin, and not by Pin1 isomerization of the AR LBD.

Pin1 inhibits β-catenin binding to the AR LBD. The most straightforward interpretation of these results was that Pin1 abrogates β-catenin coactivation of AR by acting on β-catenin to prevent its binding to the AR LBD. To test this hypothesis, we directly examined the effects of Pin1 on β-catenin binding to the AR LBD. Cell lysates from control or Pin1 transfected 293T cells (which express substantial levels of β-catenin and can be transfected at very high efficiency) were incubated with GST or GST-AR-LBD fusion proteins linked to glutathione-agarose beads, and bound β-catenin was detected by immunoblotting. As shown in figure 5C, the endogenous β-catenin bound specifically to the GST-AR LBD beads as compared to the GST control beads. However, specific binding was markedly diminished when lysates from Pin1 transfected 293T cells were analyzed. These results, in conjunction with above functional studies, indicated that Pin1 isomerization of β-catenin abrogated its coactivation of AR by inhibiting β-catenin binding to the AR LBD.

Pin1 antagonizes the inhibition of Tcf4 signaling by the DHT liganded AR. While these data showed that Pin1 could suppress β-catenin coactivation of AR activity, a function of the AR-β-catenin interaction appears to be sequestration of nuclear β-catenin and consequent inhibition of β-catenin/Tcf4 signaling (1,10,27,30,33,43,46). Therefore, further studies were carried out to determine the effect of Pin1 on AR inhibition of β-catenin/Tcf signaling. As has

been shown previously, β-catenin strongly stimulates the Tcf regulated pTopflash reporter, and this activity can be markedly repressed by AR in a dose- and DHT-dependent manner (Fig. 6A). The pTopflash reporter in 293T cells was similarly activated by the Pin1 mediated increase in β-catenin (Fig. 6B). However, in this case the AR inhibition of pTopflash activity was markedly diminished. These results indicated that Pin1 could enhance β-catenin/Tcf signaling in AR expressing cells by preventing β-catenin sequestration by AR.

Coimmunoprecipitation experiments were next carried out to directly test the hypothesis that AR can sequester β-catenin from Tcf4, and that this action can be blocked by Pin1. Lysates from transfected 293T cells were immunoprecipitated with anti-Tcf4 or control antibodies, and then immunoblotted to detect Tcf4 associated β-catenin. In cells transfected with Tcf4 alone, β-catenin was coimmunoprecipitated by anti-Tcf4, but not the control antibody (Fig. 6C and D, lane 1). In contrast, AR cotransfection caused a marked decrease in the amount of Tcf4 associated β-catenin (Fig. 6D, lane 2). Although there was also a small decrease in the level of total β-catenin and Tcf4 (Fig. 6E), this result provided direct evidence for AR sequestration of β-catenin. Importantly, cotransfection of Pin1 with AR restored the coimmunoprecipitation of β-catenin by anti-Tcf4 (Fig. 6D, lanes 3 and 4). Moreover, this was not due to an increase in Tcf4 or β-catenin, or due to a decrease in AR (Fig 6E). Taken together, these biochemical studies and the above functional data showed that Pin1 can prevent AR mediated repression of β-catenin /Tcf4 signaling by abrogating AR binding to β-catenin.

Finally, we examined LNCaP PCa cells to determine whether Pin1 could abrogate β-catenin inhibition by the endogenous AR in PTEN deficient PCa cells. LNCaP cells were transfected

with the pTopflash reporter, minus or plus Pin1, and activity of the pTopflash reporter in response to DHT was assessed. Treatment with DHT caused a rapid decline in pTopflash activity, and this inhibition was completely prevented by Pin1 (Fig. 7A). Similar results were observed in LNCaP cells stably transfected with Pin1. In control vector transfected cells, pTopflash activity was repressed by DHT. In contrast, there was no inhibition in Pin1 expressing clones (Fig. 7B). These results confirmed β-catenin inhibition by endogenous AR in PCa cells, and showed that abrogation of this inhibition is a mechanism by which Pin1 can enhance β-catenin/Tcf4 activity in PCa.

DISCUSSION

The increased expression of β-catenin plays a major role in many cancers, but its contribution to PCa and role of the AR-β-catenin interaction have not been clear. Previous transient transfection studies using Tcf4 regulated reporter genes have indicated that a function of the AR-β-catenin interaction may be to sequester limited nuclear β-catenin and thereby suppress β-catenin/Tcf4 signaling (10,30,46). Pin1 has been shown to stabilize β-catenin in cells with active APC/GSK-3β mediated β-catenin degradation, and increased Pin1 expression in radical prostatectomy specimens has been correlated with greater risk of PCa recurrence (2,42). This study found that Pin1 was markedly increased in advanced metastatic PCa, and therefore assessed increased Pin1 as a mechanism for enhanced β-catenin expression and function in PCa (and specifically in PTEN deficient PCa cells). Pin1 expression enhanced β-catenin/Tcf4 signaling in LNCaP cells, and stable expression of Pin1 in LNCaP transfectants markedly

enhanced tumor growth in immunodeficient mice. However, consistent with PTEN loss and the constitutive suppression of GSK-3β activity in these cells, increased Pin1 did not increase β-catenin levels (34,44). Instead, Pin1 abrogated the AR-β-catenin interaction and suppressed the ability of AR to antagonize β-catenin/Tcf4 activity. Taken together, these data indicate that Pin1 can stimulate β-catenin/Tcf4 signaling in PCa, including PTEN deficient prostate cancers, by abrogating AR mediated suppression of β-catenin function. These results demonstrate roles for Pin1 and β-catenin in PCa progression, and support a physiological role for the AR-β-catenin interaction in suppressing β-catenin/Tcf4 signaling.

The hypothesis that Pin1 augments β-catenin/Tcf4 signaling in PCa was supported by increased expression of *c-myc*, an endogenous β-catenin/Tcf4 target gene, in LNCaP cell lines stably transfected with Pin1. Significantly, the Pin1 stable LNCaP cell lines also had increased expression of Tcf4. The relatively low levels of endogenous Tcf4 expression in LNCaP cells, as well as weak β-catenin/Tcf4 signaling as assessed by transfection with the Tcf regulated pTopflash reporter, have been noted previously (10). As Tcf4 functions as a strong transcriptional repressor in the absence of nuclear β-catenin through recruitment of the Grouch/TLE family of corepressor proteins, there is presumably selective pressure to keep its level low in the absence of coactivation by nuclear β-catenin. Conversely, the increased availability of nuclear β-catenin in Pin1 expressing LNCaP cells likely selects for cells with increased Tcf4 levels, which can take advantage of the increased β-catenin to enhance expression of β-catenin/Tcf4 regulated genes such as *c-myc*.

Transient transfection assays showed that Pin1 prevented β-catenin coactivation of the isolated AR LBD, but did not repress the isolated AR N-terminus or the CPA liganded full length

AR (which does not recruit β-catenin). These results indicated that the Pin1 mediated isomerization of β-catenin, which blocks its interaction with APC, was similarly preventing β-catenin interaction with the AR LBD. This interpretation was supported by decreased inhibitory activity of a catalytically inactive Pin1 mutant, and by site directed mutagenesis to remove the single potential Pin1 recognition site in the AR LBD, as this did not prevent Pin1 mediated abrogation of the β-catenin-AR interaction. Direct binding studies further confirmed that Pin1 could prevent β-catenin binding to the AR LBD. Finally, β-catenin/Tcf4 coimmunoprecipitation experiments showed directly that AR could suppress β-catenin association with Tcf4, and that this suppression could be abrogated by Pin1. Interestingly, the AR may also interact with a number of other Pin1 target proteins (including c-Jun, cyclin D1, and p53), suggesting that Pin1 may further regulate AR function through modulation of interactions with additional proteins.

The WW-domain of Pin1 recruits this enzyme to pSer/pThr-Pro motifs, and proline isomerization at these sites can both regulate dephosphorylation and alter interactions with other proteins (60). Pin1 appears to bind to a pSer-Pro site in the third Armadillo repeat of β-catenin, and mutation in this serine (Ser246) can block the ability of Pin1 to prevent β-catenin-APC binding *in vitro* (42). The site on β-catenin that mediates AR binding is within the first six Armadillo repeats, indicating that Pin1 may abrogate β-catenin binding to APC and AR by altering the same site (57). Efforts have been made to directly test this hypothesis using a previously described β-catenin Ser246Ala mutant, but this mutant is expressed at extremely low levels in transient transfections and does not yield any detectable coactivation of AR or Tcf4 (data not shown) (42). Therefore, it is not yet clear whether Pin1 modulates β-catenin binding to

APC and AR via the same or distinct sites, or whether different kinases regulate Pin1 recognition of these sites. It should also be noted that further direct or indirect effects of Pin1 on AR are also possible, based on Pin1 suppression of AR activity in the absence of exogenous β-catenin (although this may in part reflect isomerization of endogenous β-catenin) and augmentation of the isolated AR N-terminus.

The levels of total and nuclear β-catenin are tightly regulated by binding to APC, which mediates GSK-3β dependent degradation of β-catenin and can also stimulate its nuclear export (16,31,36,39-41). Therefore, although Pin1 mediated abrogation of β-catenin binding to APC does not increase β-catenin stability in PTEN deficient PCa cells, it may nonetheless further increase β-catenin/Tcf4 activity by decreasing the nuclear export of β-catenin. Indeed, immunofluoresence studies indicate that Pin1 can cause a relative increase in the levels of nuclear β-catenin in LNCaP cells (data not shown). In support of the hypothesis that APC may continue to mediate nuclear export of β-catenin in advanced PCa, loss of heterozygosity in the *APC* locus, hypermethylation of the *APC* promoter, and *APC* mutations have been reported in PCa and may correlate with more advanced disease (6,15,19,26,35,58).

In a previous study we found a correlation between AR ligands that support β-catenin binding and stimulate LNCaP cell growth, and suggested that AR recruitment of β-catenin may be necessary to stimulate the expression of one or more growth promoting genes (27). In contrast, this study shows that the β-catenin-AR interaction can function to suppress β-catenin/Tcf4 signaling and tumorgenesis. Taken together, these findings suggest that the β-catenin-AR interaction may have a dual function. AR coactivation and stimulation of growth promoting genes may predominate in cells with active β-catenin degradation, while AR sequestration of

β-catenin may play an important role in suppressing the tumorgenic activity of excess free nuclear β-catenin in cells with physiological active Wnt signaling or pathological loss of regulated β-catenin degradation. In the latter cases, increased Pin1 expression would abrogate the AR sequestration of β-catenin and contribute to tumor progression.

In summary, these studies indicate that Pin1 contributes to the development of aggressive PCa by abrogating the AR-β-catenin interaction and thereby increasing β-catenin coactivation of Tcf4 and expression of Tcf4 regulated genes. These findings also strongly support a physiological role for AR in the negative regulation of β-catenin/Tcf4 signaling. Importantly, this may provide a rationale for the early use of intermittent androgen ablation therapy to suppress β-catenin function, and suggests that this therapy may eventually fail in part due to increased Pin1 expression. Finally, this study indicates that drugs targeting Pin1, or selective AR antagonists that maintain or enhance AR-β-catenin binding, may be more effective than conventional androgen ablation therapies in a subset of PCa patients.

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Table 1. Pin1 expression in benign prostate, primary and metastatic prostate cancer.

Pin1 expression	benign	primary PCa	metastatic PCa
negative staining	16	10	0
weak staining	9	14	5
moderate staining	0	6	10
strong staining	0	0	14
total samples	25	30	29

FIGURE LEGENDS

Figure 1. Expression of Pin1 and β-catenin in metastatic PCa. A-C, tissue microarrays containing benign prostate, primary PCa, and metastatic PCa samples were immunostained for Pin1. D, representative samples of Pin1 and β-catenin immunostaining in adjacent sections of benign prostate, primary PCa, and metastatic PCa.

Figure 2. Pin1 expression enhances tumor growth and β–catenin/Tcf4 activity in PTEN deficient LNCaP PCa cells. A, Pin 1 (8 mice) or vector control (4 mice) stable LNCaP cell lines were implanted subcutaneously in 50% Matrigel into the flanks of male SCID mice and the % mice with palpable tumors was determined weekly. B, Pin1 expressing LNCaP clones (P1 and P2) or control clone were lysed in digitonin buffer or in 1% SDS and lysates were immunoblotted to identify free and total β-catenin, respectively. C, control or Pin1 expressing LNCaP clones were transfected with pTopflash (50 ng) and CMV-*Renilla* (2.5 ng) reporter plasmids. Firefly versus *Renilla* luciferase activities were determined and expressed as relative light unit (RLU). D, Control and Pin1 clones in 10% FBS or in serum free medium for 24 hours were lysed in 1% SDS and immunoblotted for c-myc and Tcf4. E, RNA was extracted from control or Pin1 clones and *c-myc* gene expression was measured by quantitative real time RT-PCR.

Figure 3. Pin1 enhances Tcf4 and suppresses AR coactivation by β-catenin. CV1 cells were transfected with A, pTopflash (20 ng); B, ARE_4-Luciferase (10 ng); C, PSA-Luciferase (10 ng); or D, ERE_2-Luc (10 ng) reporters, together with Pin1 and β-catenin expression vectors as

indicated, and pRL-CMV (2.5 ng) as an internal control. B and C were cotransfected with AR, and D was cotransfected with ERα vectors (10 ng). DHT or estradiol (E2) was added at final concentrations of 10 nM as indicated. Luciferase activities were determined 24 hours after hormone treatment.

Figure 4. Pin1 represses β-catenin coactivation of the AR LBD but does not repress CPA liganded AR. A, CV1 cells were transfected with pBIND-AR-LBD (50 ng), pG5-Luciferase (10 ng), β-catenin, and Pin1 vectors. Luciferase activities were determined 24 hours after DHT treatment. B and C, CV1 cells were transfected with AR DBD-LBD (50 ng) (B) or AR N-DBD (30 ng) (C) vectors, ARE_4-Luciferase reporter (10 ng), β-catenin, and Pin1 as indicated. D and E, CV1 cells were transfected with pCIneo-AR (10 ng), ARE_4-Luciferase reporter (10 ng), and β-catenin and Pin1 expression vectors as indicated. Transfected cells were then treated for 24 hours with DHT (D) or CPA (E). pRL-CMV (2.5 ng) was used as an internal control. F, CV1 cells were transfected as above with pBIND-AR-LBD (50 ng), pG5-Luciferase (10 ng), β-catenin (50 ng), and wild-type (WT) or K63A (KA) mutant Pin1. The % inhibition of control (no Pin1) activity is shown. Pin1 immunoblots were carried out on pooled protein from the triplicate samples.

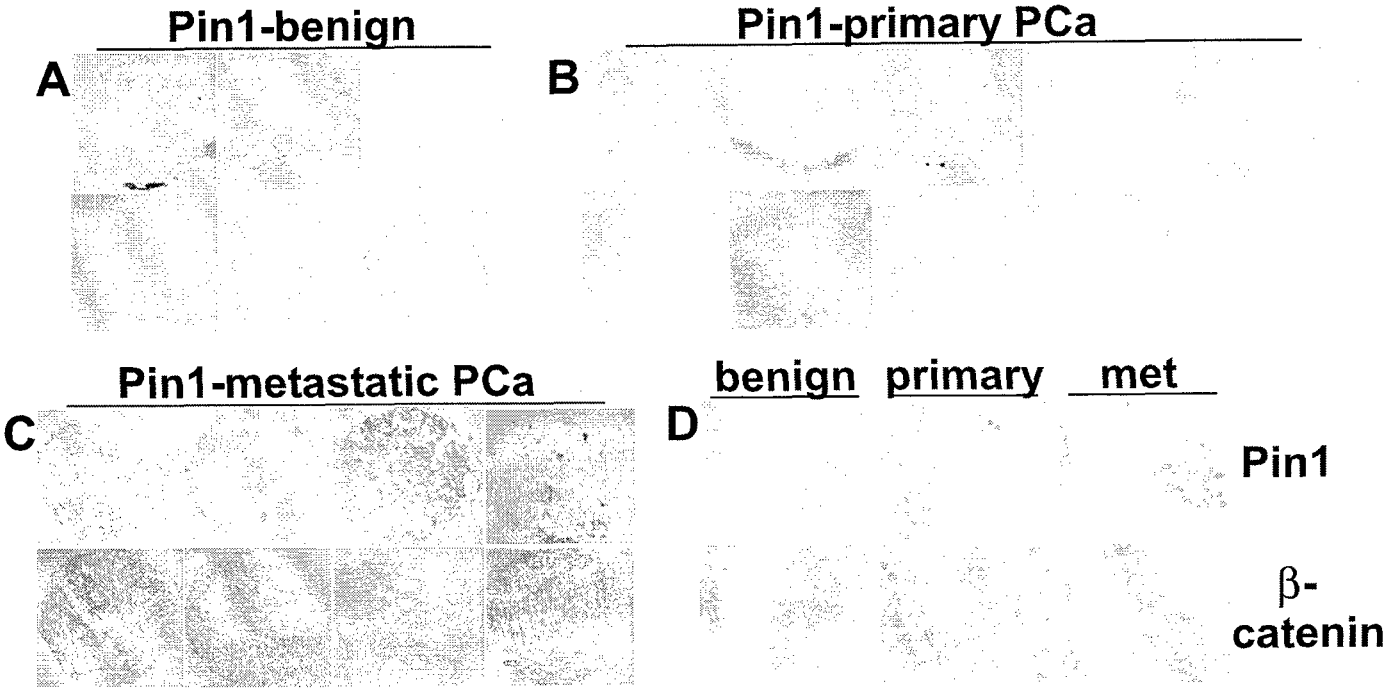
Figure 5. Inhibitory effect of Pin1 is mediated through β-catenin by disruption of its binding to the AR LBD. A and B, CV1 cells were transfected with pRL-CMV (2.5 ng), ARE_4-Luciferase (10 ng), and pCIneo-AR (wild-type AR) (A), or pCIneo-AR(Thr799Ala)(10 ng)

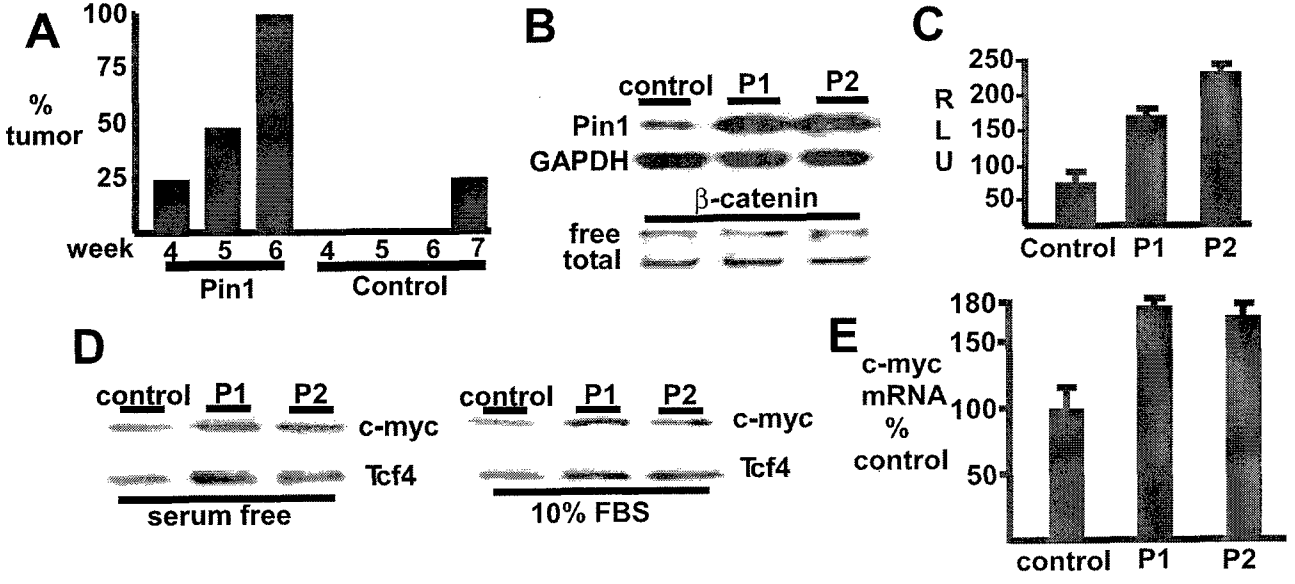
(B). Additional plasmids were cotransfected as indicated, and cells were treated with vehicle or DHT (10 nM). C, 293T cells were transfected with either 10 μg of pcDNA3.1 vector (-) or pcDNA-Pin1 (+), as indicated. Lysates were precipitated with 5 μg of GST or GST-AR LBD fusion proteins bound to glutathione agarose beads and bound β-catenin was determined by immunoblotting.

Figure 6. Pin1 antagonizes the inhibition of Tcf4 signaling by the DHT-liganded AR. A and B, 293T cells were transfected with pRL-CMV (2.5 ng), pTOPFLASH (20 ng), pCIneo-AR, and β-catenin (A) or Pin1 (B) expression vectors as indicated. C, D, and E, 293T cells were transfected with 3 μg of β-catenin and Tcf4 plasmids in every case, 3 μg of AR as indicated, and 3 or 9 μg of Pin1 vector as indicated. pcDNA3.1 vector was used to equalize the total plasmid amount. Cells lysates were precleared and then immunoprecipitated with control non-immune mouse serum (C) or mouse anti-Tcf4 antibody (D), followed by immunoblotting for β-catenin. The position of β-catenin is indicated with an arrow, while the lower band (*) is immunoglobulin dimer present in the anti-Tcf4 Ab preparation that is recognized by the secondary anti-mouse antibody alone (not shown). E, inputs (1%) for the indicated proteins.

Figure 7. Pin1 expression in LNCaP cells prevents AR mediated suppression of β-catenin/Tcf4 activity. A, LNCaP cells were transfected with pTopflash reporter (50 ng), pCMV-RL (2.5 ng), and Pin1 (10 ng) expression vectors for 24 hours as indicated, followed by another 24 hours in steroid hormone depleted medium. They were then stimulated for 1 or 2

hours with 10 nM DHT and assayed for luciferase versus *Renilla* activity. B, control or stable Pin1 expressing LNCaP cells (P1 and P2) were transfected with pTopflash (50 ng) and pCMV-RL (2.5 ng) vectors for 24 hours, and luciferase versus Renilla activities were determined after another 24 hours.


Pin1-benign
A
Pin1-primary PCa
B
Pin1-metastatic PCa
C
benign
primary
met
D
Pin1
β-
catenin


A
100
75
%
tumor
50
25
week
4
5
6
4
5
6
7
Pin1
Control
B
control
P1
P2
Pin1
GAPDH
β-catenin
free
total
C
250
R
200
L
150
U
100
50
Control
P1
P2
D
control
P1
P2
c-myc
Tcf4
serum free
control
P1
P2
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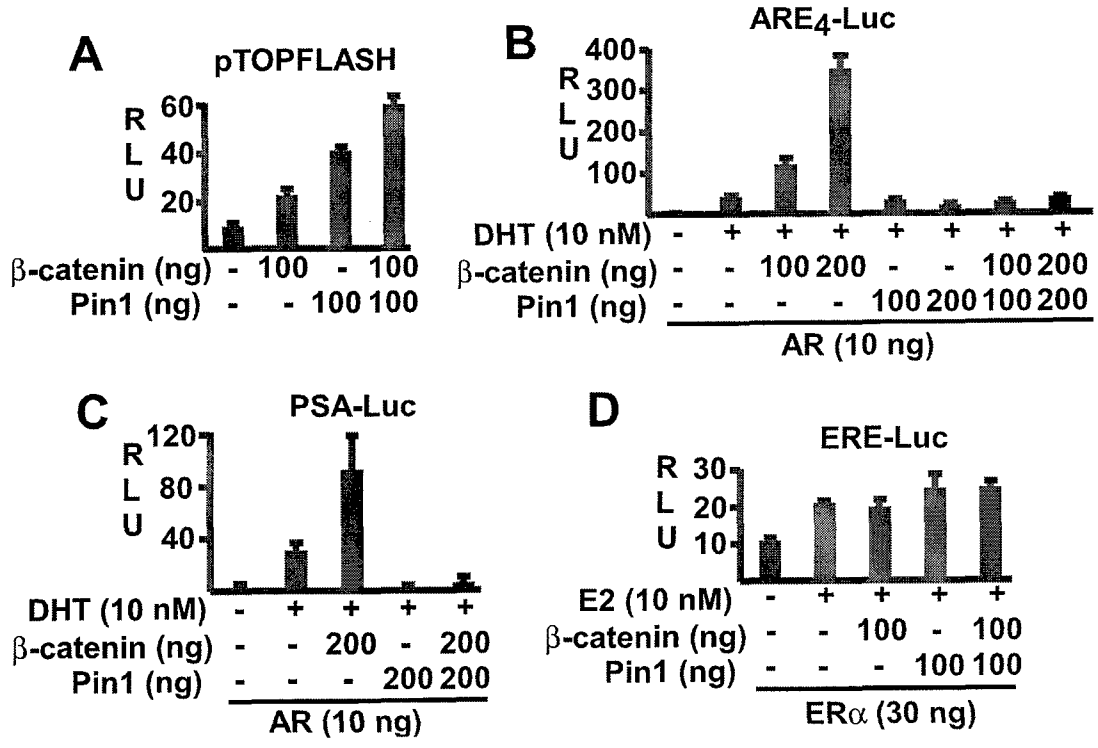
E
180
150
c-myc
mRNA
%
control
100
50
control
P1
P2


A
pTOPFLASH
RLU
60
40
20
β-catenin (ng) - 100 - 100
Pin1 (ng) - - 100 100
B
ARE4-Luc
RLU
400
300
200
100
DHT (10 nM) - + + + + + + +
β-catenin (ng) - - 100 200 - - 100 200
Pin1 (ng) - - - - 100 200 100 200
AR (10 ng)
C
PSA-Luc
RLU
120
80
40
DHT (10 nM) - + + + +
β-catenin (ng) - - 200 - 200
Pin1 (ng) - - - 200 200
AR (10 ng)
D
ERE-Luc
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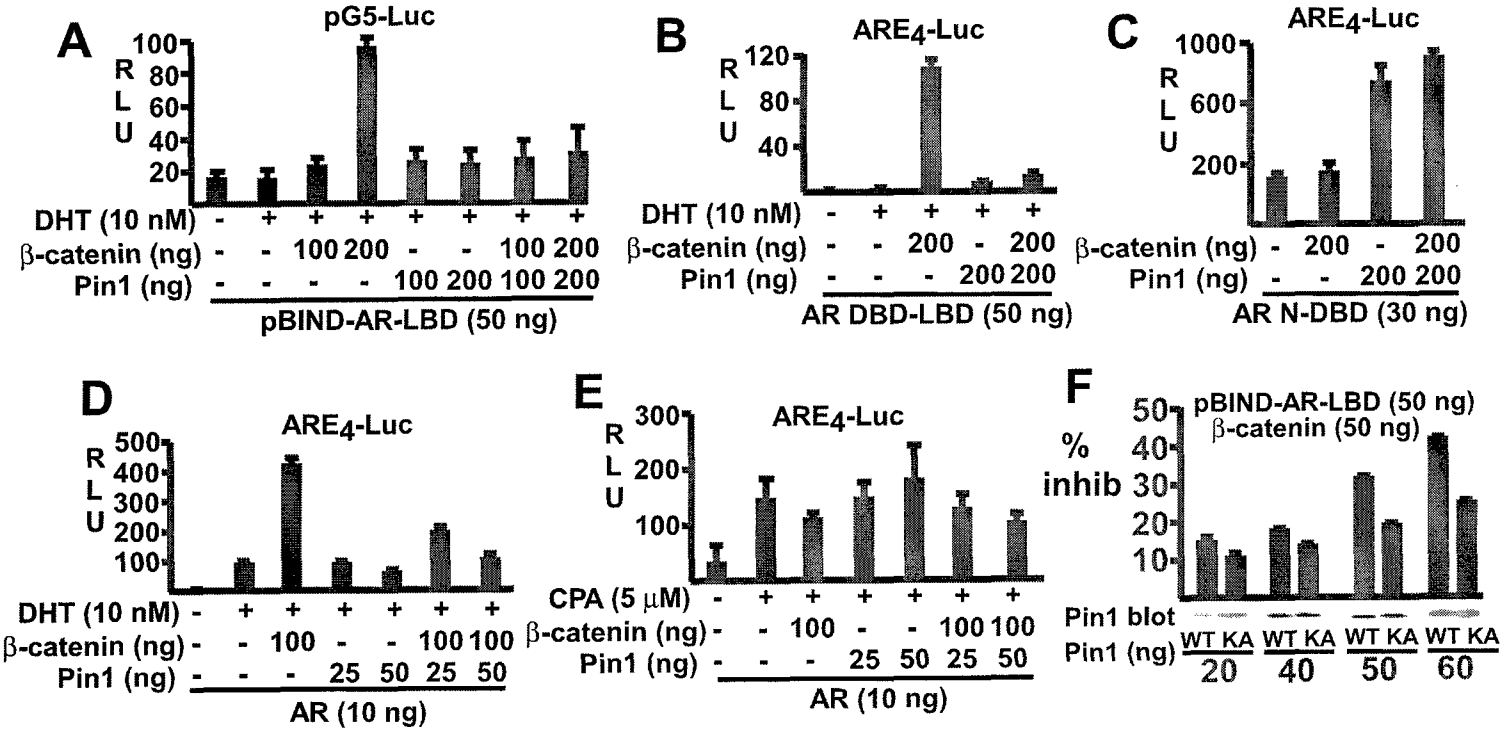
10
E2 (10 nM) - + + + +
β-catenin (ng) - - 100 - 100
Pin1 (ng) - - - 100 100
ERα (30 ng)


A
pG5-Luc
RLU
100
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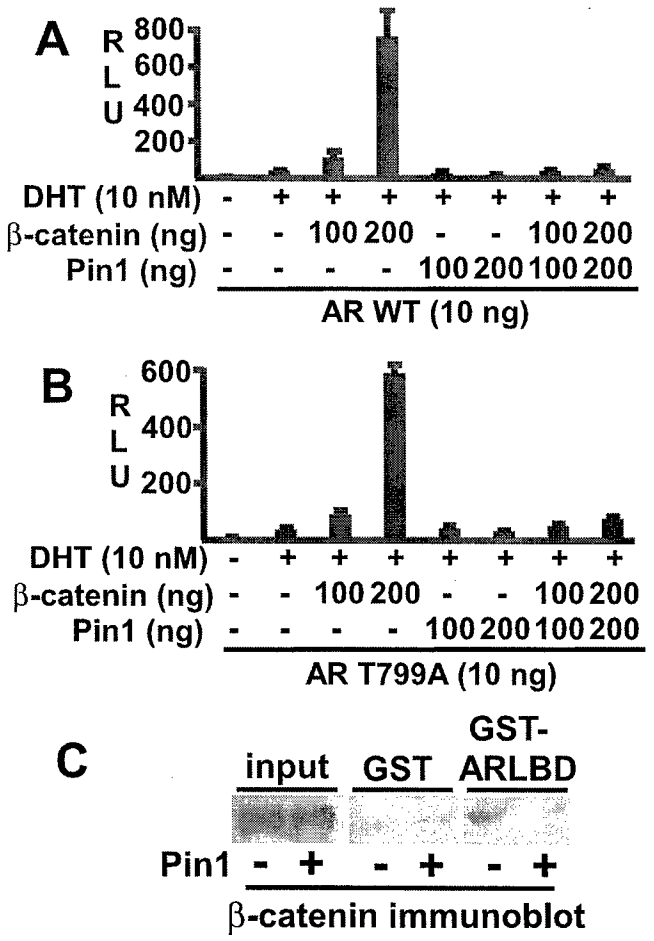
20
DHT (10 nM) - + + + + + + +
β-catenin (ng) - - 100 200 - - 100 200
Pin1 (ng) - - - - 100 200 100 200
pBIND-AR-LBD (50 ng)
B
ARE4-Luc
RLU
120
80
40
DHT (10 nM) - + + + +
β-catenin (ng) - - 200 - 200
Pin1 (ng) - - - 200 200
AR DBD-LBD (50 ng)
C
ARE4-Luc
RLU
1000
600
200
β-catenin (ng) - 200 - 200
Pin1 (ng) - - 200 200
AR N-DBD (30 ng)
D
ARE4-Luc
RLU
500
400
300
200
100
DHT (10 nM) - + + + + + +
β-catenin (ng) - - 100 - - 100 100
Pin1 (ng) - - - 25 50 25 50
AR (10 ng)
E
ARE4-Luc
RLU
300
200
100
CPA (5 μM) - + + + + + +
β-catenin (ng) - - 100 - - 100 100
Pin1 (ng) - - - 25 50 25 50
AR (10 ng)
F
pBIND-AR-LBD (50 ng)
β-catenin (50 ng)
% inhib
50
40
30
20
10
Pin1 blot
Pin1 (ng) WT KA WT KA WT KA WT KA
20 40 50 60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DHT (10 nM) - + + + + + + +
β-catenin (ng) - - 100 200 - - 100 200
Pin1 (ng) - - - - 100 200 100 200
AR WT (10 ng)
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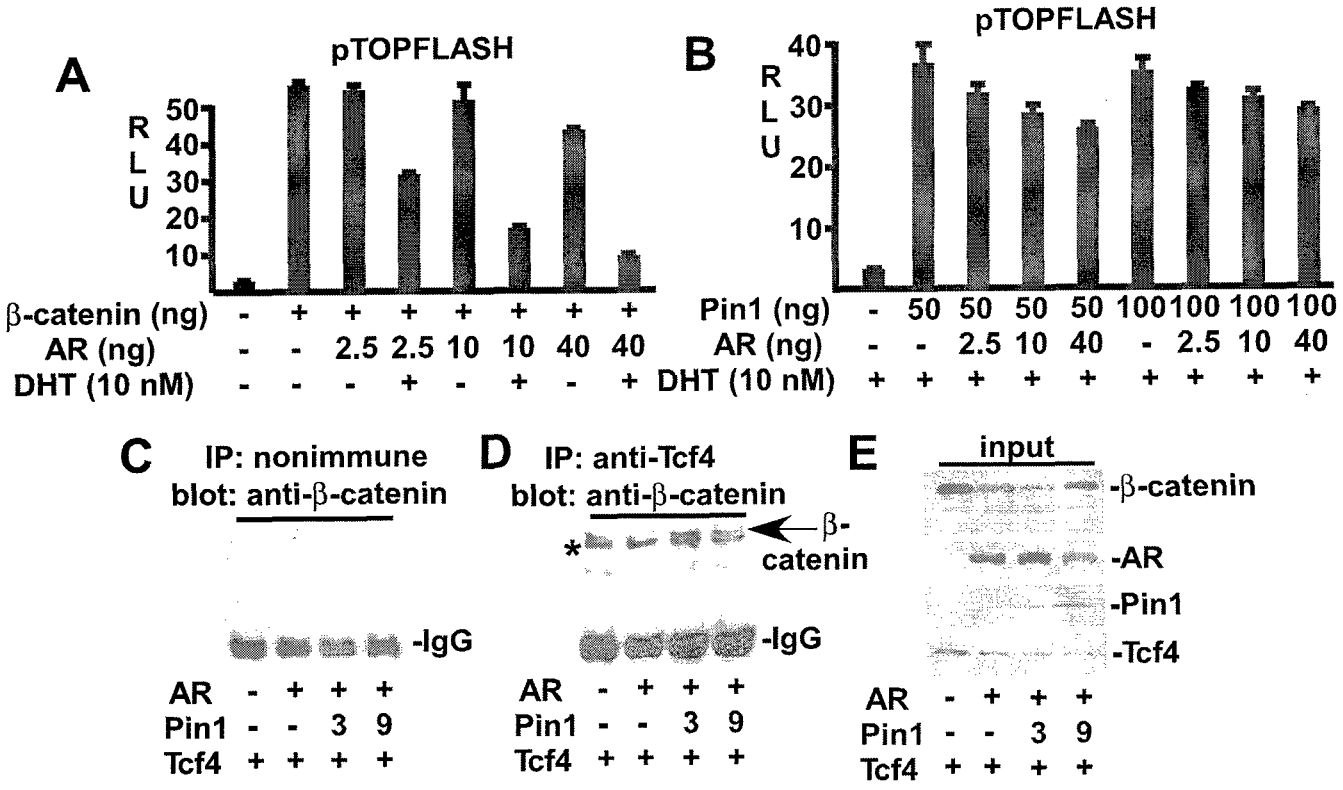
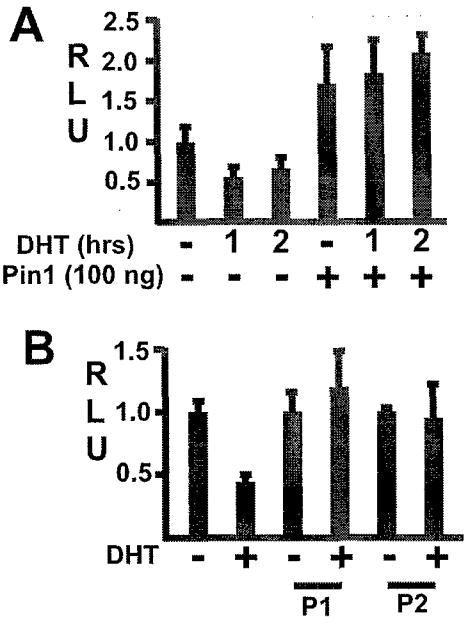
RLU
600
400
200
DHT (10 nM) - + + + + + + +
β-catenin (ng) - - 100 200 - - 100 200
Pin1 (ng) - - - - 100 200 100 200
AR T799A (10 ng)
C
input
GST
GST-ARLBD
Pin1 - + - + - +
β-catenin immunoblot


A
pTOPFLASH
RLU
50
40
30
20
10
β-catenin (ng) - + + + + + + +
AR (ng) - - 2.5 2.5 10 10 40 40
DHT (10 nM) - - - + - + - +
B
pTOPFLASH
RLU
40
30
20
10
Pin1 (ng) - 50 50 50 50 100 100 100 100
AR (ng) - - 2.5 10 40 - 2.5 10 40
DHT (10 nM) + + + + + + + + +
C
IP: nonimmune
blot: anti-β-catenin
-IgG
AR - + + +
Pin1 - - 3 9
Tcf4 + + + +
D
IP: anti-Tcf4
blot: anti-β-catenin
β-catenin
*
-IgG
AR - + + +
Pin1 - - 3 9
Tcf4 + + + +
E
input
-β-catenin
-AR
-Pin1
-Tcf4
AR - + + +
Pin1 - - 3 9
Tcf4 + + + +


A
R
L
U
2.5
2.0
1.5
1.0
0.5
DHT (hrs) - 1 2 - 1 2
Pin1 (100 ng) - - - + + +
B
R
L
U
1.5
1.0
0.5
DHT - + - + - +
P1
P2